

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :- C12N 15/12, 1/21, A61K 38/38, C12P 21/02, C12N 15/11, 1/20	A1	(11) International Publication Number: WO 95/23857
		(43) International Publication Date: 8 September 1995 (08.09.95)

(21) International Application Number: PCT/GB95/00434

(22) International Filing Date: 1 March 1995 (01.03.95)

(30) Priority Data:
9404270.2 5 March 1994 (05.03.94) GB

(71) Applicant (for all designated States except US): DELTA BIOTECHNOLOGY LIMITED [GB/GB]; Castle Court, Castle Boulevard, Nottingham NG7 1FD (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KERRY-WILLIAMS, Sean, Martin [GB/GB]; 131 Trent Road, Beeston Rylands, Nottingham NG9 1LP (GB). GILBERT, Sarah, Catherine [GB/GB]; 65 Dene Road, Headington, Oxford OX3 7EQ (GB).

(74) Agents: BASSETT, Richard et al.; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published

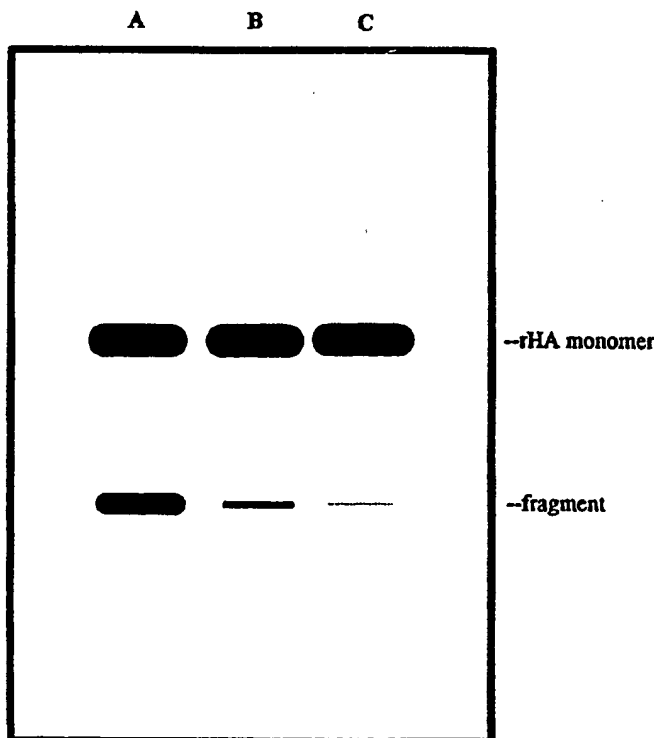
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: YEAST STRAINS AND MODIFIED ALBUMINS

(57) Abstract

Albumin, for example human albumin, is expressed and secreted in yeast which has been mutated to lack the yeast aspartyl protease 3 (Yap3p) or its equivalent, thereby reducing the production of a 45kD albumin fragment. A further reduction is achieved by additionally deleting the Kex2p function. Alternatively, a modified albumin is prepared which is not susceptible to Yap3p cleavage, for example human albumin which is R410A, K413Q and K414Q.



Best Available Copy

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

YEAST STRAINS AND MODIFIED ALBUMINS

Field of the invention

- 5 The present invention relates to the production of recombinant human albumin (rHA) by yeast species.

Background and prior art

- 10 Human serum albumin (HSA) is a protein of 585 amino acids that is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The
15 production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

- In recent years yeast species have been widely used as a host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992),
20 including rHA (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for production of secreted as well as cytosolic proteins.

- 25 When *S. cerevisiae* is utilised to produce rHA, the major secreted protein is mature 67kDa albumin. However, a 45kDa N-terminal fragment of rHA is also observed (Sleep *et al*, 1990). A similar fragment is obtained when rHA is expressed in *Kluyveromyces* sp. (Fleer *et al*, 1991) and *Pichia pastoris* (EP 510 693). The fragment has the same N-terminal amino acid sequence as
30 mature rHA, but the carboxy terminus is heterogeneous and occurs between

mutagenesis of the codon for Lys⁴¹⁴, resulted in reduction in the amount of the fragment.

There is a vast array of yeast proteases which could, in principle, be degrading a desired protein product, including (in *S. cerevisiae*) yscA, yscB, yscY, yscS, other vacuolar proteinases, yscD, yscE, yscF (equivalent to kex2p), ysc α , yscIV, yscG, yscH, yscJ, yscE and kex1.

Bourbonnais *et al* (1991) described an *S. cerevisiae* endoprotease activity specific for monobasic sites, an example of which (Arg⁴¹⁰) exists in this region of albumin. This activity was later found to be attributable to yeast aspartyl protease 3 (Yap3) (Bourbonnais *et al*, 1993), an enzyme which was originally described by Egel-Mitani *et al* (1990) as an endoprotease similar to Kex2p in specificity, in that it cleaved at paired basic residues. Further work suggested that Yap3p is able to cleave monobasic sites and between, and C-terminal to, pairs of basic residues, but that cleavage at both types of sites is dependent on the sequence context (Azaryan *et al*, 1993; Cawley *et al*, 1993).

As already discussed, the region of the C-terminus of rHA fragment contains both a monobasic (Arg⁴¹⁰) and a dibasic site (Lys⁴¹³Lys⁴¹⁴). However, even though a Kex2p-like proteolytic activity is present in human cells and is responsible for cleavage of the pro sequence of HSA C-terminal to a pair of arginine residues, the fragment discussed above is not known to be produced in humans. This indicates that the basic residues Arg⁴¹⁰, Lys⁴¹³ and Lys⁴¹⁴ are not recognised by this Kex2p-like protease, in turn suggesting that this region of the molecule may not be accessible to proteases in the secretory pathway. Thus, the Yap3p protease could not have been predicted to be responsible for the production of the 45kDa fragment. In addition, Egel-Mitani *et al* (1990 *Yeast* 6, 127-137) had shown Yap3p to be similar to Kex2p in cleaving the MF α propheromone. Since removal of the Kex2p function alone does not

reduce the amount of the fragment produced, there was no reason to suppose that removal of the Yap3p function would be beneficial. Indeed, Bourbonnais *et al* (1993) showed that *yap3* strains had a decreased ability to process pro-somatostatin, and therefore taught away from using *yap3* strains in the production of heterologous proteins.

Summary of the invention

The solution to the problem identified above is, in accordance with the invention, to avoid or at least reduce production of the fragment in the initial fermentation, rather than to remove it during purification of the albumin. We have now found that, out of the 20 or more yeast proteases which are so far known to exist, it is in fact the Yap3p protease which is largely responsible for the 45kD fragment of rHA produced in yeast. The present invention provides a method for substantially reducing the amount of a 45kDa fragment produced when rHA is secreted from yeast species. The reduction in the amount of fragment both improves recovery of rHA during the purification process, and provides a higher quality of final product. A further, and completely unexpected, benefit of using *yap3* strains of yeast is that they can produce 30-50% more rHA than strains having the Yap3p function. This benefit cannot be accounted for merely by the reduction of rHA fragment from ~15% to 3-5%.

Thus, one aspect of the present invention provides a process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

Preferably, the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.

Suitably, the yeast is *S. cerevisiae* which lacks a functional *YAP3* gene. However, the invention is not limited to the use of *S. cerevisiae*, since the problem of 45 kDa fragment production is found also in other yeast genera, for example *Pichia* and *Kluyveromyces*, which shows that they have equivalent proteases (ie Yap3p proteolytic activity); see Clerc *et al* (1994), page 253. We have confirmed this by hybridisation analysis to locate homologues of Yap3p in non-*Saccharomyces* genera. A gene is regarded as a homologue, in general, if the sequence of the translation product has greater than 50% sequence identity to Yap3p. In non-*Saccharomyces* genera, the Yap3p-like protease and its gene may be named differently, but this does not of course alter their essential nature.

The level of fragment can be reduced still further if, as well as substantially eliminating the Yap3p proteolytic activity, the Kex2p function is also substantially eliminated even though, as mentioned above, elimination of the Kex2p function alone does not affect the level of fragment. As in the case of Yap3p, the Kex2p function is not restricted to *Saccharomyces*; see Gellissen *et al* (1992), especially the sentence bridging pages 415 and 416, showing that *Pichia* has a Kex2p function. The genes encoding the Kex2p equivalent activity in *Kluyveromyces lactis* and *Yarrowia lipolytica* have been cloned (Tanguy-Rougeau *et al*, 1988; Enderlin & Ogrydziak, 1994).

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease (Rothstein, 1983). Alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations by the process of transplacement (Winston *et al*, 1983). Preferably, the activity of the enzyme is reduced to at most 50% of the wild-type level, more preferably no more than 25%, 10% or 5%, and most preferably is undetectable. The level of Yap3p proteolytic

activity may be measured by determining the production of the 45 kDa fragment, or by the ^{125}I - β_{h} -lipoprotein assay of Azaryan *et al* (1993), also used by Cawley *et al* (1993). Kex2p proteolytic activity may similarly be measured by known assays, for example as set out in Fuller *et al* (1989).

5

The albumin may be a human albumin, or a variant thereof, or albumin from any other animal.

10

By "variants" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin which include the region cleaved by Yap3p, for example those fragments disclosed in EP 322 094 (namely HSA (1-n), where n is 369 to 419) which are sufficiently long to include the Yap3p-cleaved region (ie where n is 403 to 419); and fusions of albumin (or Yap3p-cleavable portions thereof) with other proteins, for example the kind disclosed in WO 90/13653.

15

20 By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and site-directed mutagenesis as described below.

25

A second aspect of the invention provides a modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with the *S. cerevisiae* yeast aspartyl protease 3 (Yap3p) when expressed in yeast, characterised in that the
30 modified albumin is not susceptible to such cleavage.

Preferably, the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein. Suitably, the said monobasic amino acid is arginine. Conveniently, the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin, especially any of
5 Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg. Thus, in one particular embodiment, the naturally-occurring albumin is human albumin and the modified protein lacks Arg⁴¹⁰ and, optionally, one or both Lys⁴¹³Lys⁴¹⁴ lysines. For example, the modified albumin may be human albumin having the amino acid changes R410A, K413Q, K414Q. Equivalent modifications in bovine
10 serum albumin include replacing the Arg⁴⁰⁸ and/or one or both of Arg⁴¹¹Lys⁴¹². The person skilled in the art will be able to identify monobasic sites and pairs of basic residues in other albumins without difficulty.

The numbering of the residues corresponds to the sequence of normal mature
15 human albumin. If the albumin is a variant (for example a polymorphic form) having a net deletion or addition of residues N-terminal to the position identified, then the numbering refers to the residues of the variant albumin which are aligned with the numbered positions of normal albumin when the two sequences are so aligned as to maximise the apparent homology.

20

A third aspect of the invention provides a polynucleotide encoding such a modified albumin.

The DNA is expressed in a suitable yeast (either the DNA being for a modified
25 albumin, or the yeast lacking the Yap3p function) to produce an albumin. Thus, the DNA encoding the albumin may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate yeast cell for the expression and production of the albumin.

30

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

5

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

10

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the albumin, which can then be recovered, as is known.

15

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps). Other yeast expression plasmids are disclosed in EP-A-258 067, EP-A-286 424 and EP-A-424 117.

20

The polynucleotide coding sequences encoding the modified albumin of the invention may have additional differences to those required to produce the modified albumin. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the albumin or which may improve its activity

30

or immunogenicity, as well as reducing its susceptibility to a Yap3p protease activity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle
 5 (1985). Since such modified coding sequences can be obtained by the application of known techniques to the teachings contained herein, such modified coding sequences are within the scope of the claimed invention.

Exemplary genera of yeast contemplated to be useful in the practice of the
 10 present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula* (now reclassified as *Pichia*), *Histoplasma*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of
 15 *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* sp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* sp. are *K. fragilis* and *K. lactis*. Examples of *Hansenula* (*Pichia*) sp. are *H. polymorpha* (now *Pichia angusta*), *H. anomala* (now *P. anomala*) and *P. pastoris*. *Y. lipolytica* is an example of a suitable *Yarrowia*
 20 species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. Suitable promoters for *S. cerevisiae* include those associated with
 25 the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and hybrid
 30 promoters involving hybrids of parts of 5' regulatory regions with parts of 5'

regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are
5 the thiamine-repressible promoter from the *nmt* gene as described by Maundrell
(1990) and the glucose-repressible *fbp1* gene promoter as described by Hoffman
& Winston (1990).

Methods of transforming *Pichia* for expression of foreign genes are taught in,
10 for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857
467, incorporated herein by reference), and *Pichia* expression kits are
commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen
Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

15 The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J.*
Gen. Microbiol. 132, 3459-3465 include information on *Hansenula* vectors and
transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991,
Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how
to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being
20 *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of
a eukaryotic gene which contains proper signals for transcription termination
and polyadenylation. Suitable 3' flanking sequences may, for example, be
25 those of the gene naturally linked to the expression control sequence used, ie
may correspond to the promoter. Alternatively, they may be different in which
case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The albumin is initially expressed with a secretion leader sequence, which may
30 be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae*

include that from the mating factor α polypeptide (MF α -1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. When the yeast strain lacks Kex2p activity (or equivalent) as well as being *yap3*, it may
 5 be advantageous to choose a secretion leader which need not be cleaved from the albumin by Kex2p. Such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MF α -1, β -glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α -galactosidase (*MEL1*); *K. lactis*
 10 killer toxin; and *Candida* glucoamylase.

Various non-limiting embodiments of the invention will now be described by way of example and with reference to the accompanying drawings in which:

15 Figure 1 is a general scheme for the construction of mutated rHA expression plasmids, in which HA is a human albumin coding sequence, L is a sequence encoding a secretion leader, P is the PRB1 promoter, T is the ADH1 terminator, amp is an ampicillin resistance gene and LEU2 is the leucine selectable marker;

20

Figure 2 is a drawing representing a Western blot analysis of mutant rHA secreted by *S. cerevisiae*, in which Track A represents the culture supernatant from DB1 cir^o pAYE316 (normal rHA), Track B represents the culture supernatant from DB1 cir⁺ pAYE464 (alteration 1), and Track C represents the
 25 culture supernatant from DB1 cir⁺ pAYE468 (alteration 3);

Figure 3 is a scheme of the construction of pAYE515;

Figure 4 is a comparison of rHA fragment production by wild-type and
 30 protease-disrupted strains, presented as a drawing of an anti-HSA Western blot

of culture supernatant from shake flask cultures separated by non-reducing 10% SDS/PAGE, in which Track A corresponds to DB1 cir^o pAYE316, Track B corresponds to DXY10 cir^o pAYE316 (*yap3* strain), and Track C corresponds to ABB50 cir^o pAYE316 (*yap3*, *kex2* strain);

5

Figure 5 is similar to Figure 4 but shows Coomassie Brilliant Blue stained 12.5% SDS Phastgel (Pharmacia) of culture supernatants from fed batch fermentations, namely Track D for the HSA standard, Track E for DB1 cir^o pAYE316, Track F for DB1 $\Delta kex2$ cir^o pAYE522, and Track G for DXY10 cir^o pAYE522; and

10

Figure 6 is a scheme for the construction of pAYE519.

Detailed description of the invention

15

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding HSA are derived from the cDNA disclosed in EP 201 239.

20 **Example 1: Modification of the HSA cDNA.**

In order to investigate the role of endoproteases in the generation of rHA fragment, the HSA cDNA (SEQ1 (which includes a sequence encoding the artificial secretion leader sequence of WO 90/01063)) was modified by site-directed mutagenesis. Three separate changes were made to the HSA sequence (SEQ2). The first, using the mutagenic primer FOG1, changed the Arg⁴¹⁰ codon only, replacing it with an Ala codon, leaving intact the dibasic site, Lys⁴¹³Lys⁴¹⁴. The second change, using primer FOG2, changed the residues 407-409, including the C-terminal residues of fragment, from LeuLeuVal to AlaValAla. The third change, using the primer FOG3, altered residues 410-

30

414 from ArgTyrThrLysLys (SEQ3) to AlaTyrThrGlnGln (SEQ4). The oligonucleotides encoded not only the amino acid changes, but also conservative base changes that create either a *PvuII* or an *SpeI* restriction site in the mutants to facilitate detection of the changed sequences.

5

Single-stranded DNA of an M13mp19 clone, mp19.7 (EP 201 239; Figure 2), containing the HSA cDNA was used as the template for the mutagenesis reactions using the *In Vitro* Mutagenesis System, Version 2 (Amersham International plc) according to the manufacturer's instructions. Individual
 10 plaques were selected and sequenced to confirm the presence of the mutations. Double stranded RF DNA was then made from clones with the expected changes and the DNA bearing the mutation was excised on an *XbaI/SacI* fragment (Figure 1). This was used to replace the corresponding wild-type fragment of pAYE309 (EP 431 880; Figure 2). The presence of the mutated
 15 *XbaI/SacI* fragment within the plasmid was checked by digesting with *PvuII* or *SpeI* as appropriate. These *HindIII* fragments were excised and inserted into the expression vector pAYE219 (Figure 1) to generate the plasmids pAYE464 (alteration 1, R410A), pAYE470 (alteration 2, L407A, L408V, V409A) and pAYE468 (alteration 3, R410A, K413Q, K414Q). These expression plasmids
 20 comprise the *S. cerevisiae PRB1* promoter (WO 91/02057) driving expression of the HSA/MF α 1 leader sequence (WO 90/01063) fused in-frame with the mutated HA coding sequence which is followed by the *ADHI* transcription terminator. The plasmids also contain part of the 2 μ m plasmid to provide replication functions and the *LEU2* gene for selection of transformants.

25

pAYE464, pAYE470 and pAYE468 were introduced into *S. cerevisiae* DB1 cir⁺ (*a*, *leu2*; Sleep *et al*, 1990) by transformation and individual transformants were grown for 3 days at 30°C in 10ml YEPS (1% w/v yeast extract, 2% w/v peptone, 2% w/v sucrose) and then the supernatants were examined by anti-HSA
 30 Western blot for the presence of the rHA fragment. The Western blots clearly

showed that fragment was still produced by the strains harbouring pAYE464, although the level was reduced slightly compared to the control expressing wild-type rHA. The mutations in the plasmid pAYE470 appeared to have no effect on the generation of fragment. However, DB1 cir⁺ pAYE468 showed a novel pattern of HSA-related bands, with little or no fragment.

One example of each of DB1 cir⁺ pAYE464 and DB1 cir⁺ pAYE468 were grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 ± 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH ₂ PO ₄	114.0
MgSO ₄	12.0
CaCl ₂ .6H ₂ O	3.0
Na ₂ EDTA	2.0

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
ZnSO ₄ .7H ₂ O	3.0
FeSO ₄ .7H ₂ O	10.0
MnSO ₄ .4H ₂ O	3.2
CuSO ₄ .5H ₂ O	0.079
H ₃ BO ₃	1.5
KI	0.2
Na ₂ MoO ₄ .2H ₂ O	0.5
CoCl ₂ .6H ₂ O	0.56
H ₃ PO ₄	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, (NH₄)₂SO₄ 5 g/L, citric acid monohydrate 6.09 g/L, Na₂HPO₄ 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun,

Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h⁻¹). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved. The culture supernatants were compared with those of a wild-type rHA producer by Coomassie-stained SDS/PAGE and by Western blot. These indicated (Figure 2) that, whilst elimination of the monobasic Arg⁴¹⁰ (pAYE464) did reduce the level of the fragment by a useful amount, removal of both potential protease sites (pAYE468) almost abolished the 45kDa fragment.

The above data suggested that the generation of rHA fragment might be due to endoproteolytic attack, though the absence of an effect of removal of the potential Kex2p site Lys⁴¹³Lys⁴¹⁴ (Sleep *et al*, 1990, and confirmed by other studies not noted here) unless combined with elimination of Arg⁴¹⁰, had suggested a complex etiology. The reduction in the amount of fragment with the mutated rHA could in principle be due to an effect of the changes on the kinetics of folding of the molecule and not due to the removal of protease cleavage sites.

Example 2: Disruption of the *YAP3* gene.

The *YAP3* gene encoding yeast aspartyl protease 3 was mutated by the process of gene disruption (Rothstein 1983) which effectively deleted part of the *YAP3* coding sequence, thereby preventing the production of active Yap3p.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the *YAP3* gene (Egel-Mitani *et al*, 1990) were synthesised using an Applied

Biosystems 380B Oligonucleotide Synthesiser. To assist the reader, we include as SEQ15 the sequence of the *YAP3* gene, of which 541-2250 is the coding sequence.

5 5' end

YAP3A: 5'-CGTCAGACCTTGCATGCAGCCAAGACACCCTCACATAGC-3' (SEQ5)
 YAP3B: 5'-CCGTTACGTTCTGTGGTGGCATGCCCACTTCCAAGTCCACCG-3' (SEQ6)

10

3' end

YAP3C: 5'-GCGTCTCATAGTGGAAAAGCTTCTAAATACGACAACTTCCCC-3' (SEQ7)
 YAP3D: 5'-CCCAAAATGGTACCTGTGTTCATCACTCGTTGGGATAATACC-3' (SEQ8)

15

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *YAP3* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc). Conditions were as follows: 2.5µg/ml genomic DNA, 5µg/ml of each primer, 20 denature at 94°C 61 seconds, anneal at 37°C 121 secs, extend at 72°C 181 secs for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size. The 5' fragment 25 was digested with *SphI* and cloned into the *SphI* site of pUC19HX (pUC19 lacking a *HindIII* site) to give pAYE511 (Figure 3), in which the orientation is such that *YAP3* would be transcribed towards the *KpnI* site of the pUC19HX polylinker. The 3' *YAP3* fragment was digested with *HindIII* and *Asp718* (an isoschizomer of *KpnI*) and ligated into pUC19 digested with *HindIII*/*Asp718* to 30 give pAYE512. Plasmid DNA sequencing was carried out on the inserts to confirm that the desired sequences had been cloned. The *HindIII*/*Asp718* fragment of pAYE512 was then subcloned into the corresponding sites of pAYE511 to give pAYE513 (Fig 3), in which the 5' and 3' regions of *YAP3*

are correctly orientated with a unique *Hind*III site between them. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hind*III fragment and then inserted into this site to give pAYE515 (Fig 3), with *URA3* flanked by the 5' and 3' regions of *YAP3*, and transcribed in the opposite direction to *YAP3*.

5

A *ura3* derivative of strain DB1 *cir*^o pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). The strain was grown overnight in 100 mL buffered minimal medium and the cells were collected by centrifugation and
10 then washed once with sterile water. The cells were then resuspended in 10 mL sterile water and 2 mL aliquots were placed in separate 15 mL Falcon tubes. A 5 mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0 μ L, 20 μ L, 40 μ L, 80 μ L or 160 μ L. The cells were then incubated at 30°C for 30 min and then centrifuged
15 and washed three times with sterile water. Finally, the cells were resuspended in 1 mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which
20 gave approximately 50% survival were grown on YEP plates containing 2% w/v sucrose and then replica-plated onto YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1 mg/mL) and uracil (50 μ g/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation
25 and that this defect could be corrected by introduction of the *URA3* gene by transformation. One such strain, DBU3 *cir*^o pAYE316, was transformed with the *Sph*I/*Asp*718 *YAP3-URA3-YAP3* fragment of pAYE515 with selection for *Ura*⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *YAP3* gene and
30 confirmed the disruption of the *YAP3* gene. An anti-HSA Western blot of

YEPS shake-flask supernatants of two transformants indicated that disruption of *YAP3* markedly reduced rHA fragment levels.

One *yap3* derivative of DBU3 cir^o pAYE316, designated DXY10 cir^o pAYE316, was grown several times by fed-batch fermentation in minimal medium to high cell dry weight. When supernatants were examined by Coomassie-stained PAGE and anti-HSA Western blot (Figs 4 and 5), the reduction in the level of rHA 45kDa fragment was clearly apparent; estimates of the amount of the degradation product vary from $\frac{1}{3}$ to $\frac{1}{5}$ of the levels seen with the *YAP3* parent. The amount of rHA produced was not adversely affected by the *yap3* mutation, indeed DXY10 cir^o pAYE316 was found to produce 30-50% more rHA than the *YAP3* equivalent, DB1 cir^o pAYE316. Despite the fact that cleavage of the leader sequence from the HA sequence is C-terminal to a pair of basic residues, the rHA was found to have the correct N-terminus.

The fermentation broth was centrifuged to remove the cells and then subject to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0, at which point it was >95% pure. It may be purified further by techniques known in the art.

The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

Example 3: Disruption of the *KEX2* gene in a *yap3* strain.

To construct a strain lacking both Yap3p and Kex2p activity, a *lys2* derivative of yeast strain DXY10 *cir*^o (pAYE316) was obtained by random chemical mutagenesis and selection for resistance to α -amino adipate (Barnes and Thorner, 1985). Cells were mutagenised as in Example 2 and then plated on YNB minimal medium containing 2% w/v sucrose and supplemented with 2 mg/mL DL- α -amino adipate as the sole nitrogen source and 30 μ g/mL lysine. Colonies able to grow on this medium were purified and tested to verify that they were unable to grow in the absence of lysine supplementation and that this defect could be corrected by the introduction of the *LYS2* gene by transformation. This strain was then mutated by the process of gene disruption which effectively disrupted part of the *KEX2* coding sequence, thereby preventing production of active Kex2p. To assist the reader, the sequence of the *KEX2* gene is reproduced herein as SEQ14, of which 1329-3773 is the coding sequence.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the *KEX2* gene (Fuller *et al*, 1989) were synthesised using an Applied Biosystems 380B Oligonucleotide Synthesiser.

5' end

KEX2A: 5'-CCATCTGGATCCAATGGTGCTTTGGCCAAATAAATAGTTTCAGC-3' (SEQ9)
 KEX2B: 5'-GCTTCTTTTACCGGTAACAAGCTTGAGTCCATTGG-3' (SEQ10)

3' end

KEX2C: 5'-GGTAAGGTTTAGTCGACCTATTTTTTGTGTTTGTCTGC-3' (SEQ11)
 KEX2D: 5'-GGAAACGTATGAATTCGATATCATTGATACAGACTCTGAGTACG-3' (SEQ12)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *KEX2* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories Inc). Conditions were as follows: 2.5 µg/ml genomic DNA, 5 µg/ml of each primer, denature 94°C 61s, anneal 37°C 121s, extend 72°C 181s for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size (0.9 kb for the 5' product and 0.62 kb for the 3' product). The 5' product was digested with *Bam*HI and *Hind*III and the 3' product was digested with *Hind*III and *Sal*I and then the two fragments were together cloned into pUC19HX digested with *Bam*HI and *Sal*I. A 4.8 kb *Hind*III fragment comprising the *S. cerevisiae* *LYS2* gene (Barnes & Thorner, 1985) was then inserted into the resulting plasmid at *Hind*III (ie between the two *KEX2* fragments) to form pAYE519 (Fig 6).

15

The *lys2* derivative of DXY10 cir^o (pAYE316), *lys2-16*, was transformed with the 6.0 kb *KEX2-LYS2-KEX2* fragment of pAYE519, selecting for Lys⁺ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *KEX2* gene and confirmed the disruption of the *KEX2* gene. An anti-HSA Western blot of YEPS shake-flask culture supernatants of these transformants indicated that disruption of *KEX2* in a *yap3* strain reduced the level of rHA fragment still further, despite the lack of an effect of disruption of *KEX2* alone in Example 4 below. Analysis of the rHA produced by one such strain, ABB50, indicated that the leader sequence was incorrectly processed, leading to an abnormal N-terminus.

25

The strain ABB50 (pAYE316) was cured of its plasmid (Sleep *et al*, 1991) and transformed with a similar plasmid, pAYE522, in which the hybrid leader sequence was replaced by the *S. cerevisiae* invertase (*SUC2*) leader sequence

30

This Page Blank (uspto)

5 Invertase leader HSA

Example 4: Disruption of the *KEX2* gene alone (Comparative Example).

Example 5: Identification of equivalent protease in *Pichia*.

25 As noted above, non-*Saccharomyces* yeast similarly produce the undesirable fragment of rHA and therefore have the Yap3p proteolytic activity. We have confirmed this by performing Southern hybridisations of *Pichia angusta* DNA, using the *S. cerevisiae* YAP3 gene as a probe. A specific DNA fragment was identified, showing that, not only is the Yap3p proteolytic activity present in
30 *P. angusta*, but a specific homologue of the YAP3 gene is present also.

The method of Southern hybridization used for detection of the *YAP3* homologue can be adapted to clone the gene sequence from a genomic DNA library of *Pichia* DNA using standard procedures (Sambrook *et al*, 1989). Disruption of the *YAP3* homologue in *Pichia sp.* can be achieved using similar
5 techniques to those used above for *Saccharomyces* (Cregg and Madden, 1987).

References (all incorporated by reference)

- Azaryan, A.V. *et al* (1993) *J. Biol. Chem.* **268**, 11968-11975.
- Barnes, D.A. and Thorner, J. (1985) *In* Gene Manipulations in Fungi (Bennett, J.W. and Lasure, L.L., eds), pp. 197-226, Academic Press.
- 5 Boeke, J.D. *et al* (1987) *Methods Enzymol.* **154**, 164-175.
- Botstein, D. *et al* (1979) *Gene* **8**, 17-24.
- Botstein & Shortle (1985) *Science* **229**, 193-210.
- Bourbonnais, Y. *et al* (1991) *J. Biol. Chem.* **266**, 13203-13209.
- 10 Bourbonnais, Y. *et al* (1993) *EMBO J.* **12**, 285-294.
- Cawley, N.X. *et al* (1993) *FEBS Lett.* **332**, 273-276.
- Clerc *et al* (1994) *J. Chromat. B.* **662**, 245-259.
- Collins, S.H. (1990) *In* Protein Production by Biotechnology (Harris, T.J.R., ed) pp. 61-77, Elsevier Science Publishers, Barking, Essex.
- 15 Cregg, J.M. and Madden, K.R. (1987) *In* Biological Research on Industrial Yeasts, Vol. II, Stewart, G.G., Russell, I., Klein, R.D. and Hiebsch, R.R. (Eds) CRC Press, Boca Raton, FL.
- Cregg *et al* (1993) *Bio/Technology* **11**, 905-910.
- Curling (1980) "Albumin Purification by Ion Exchange Chromatography", in
- 20 "Methods of Plasma Protein Purification", Ed. Curling, J.M., Academic Press, London.
- Enderlin, C.S. & Ogrydziak, D.M. (1994) *Yeast* **10**, 67-79.
- Fleer, R. *et al* (1991) *Bio/Technology* **9**, 968-975.
- Fuller, R.S. *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1434-1438.
- 25 Geisow, M.J. *et al* (1991) *In* Techniques in Protein Chemistry II, pp. 567-572, Academic Press, Inc.
- Gellissen *et al* (1992) *Tibtech* **10**, 413-417.
- Hoffmann & Winston (1990) *Genetics* **124**, 807-816.
- Maundrell (1990) *J. Biol. Chem.* **265**, 10857-10864.
- 30 Maurel *et al* (1989) "Biotechnology of Plasma Proteins", *Colloque INSERM*

175, 19-24.

Romanos, M.A. (1992) *Yeast* 8, 423-488.

Rothstein, R.J. (1983) *Methods Enzymol.* 101, 203-211.

Sambrook, J. *et al* (1989) *Molecular Cloning: a Laboratory Manual*, 2nd
5 edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sleep, D. *et al* (1990) *Bio/Technology* 8, 42-46.

Sleep, D. *et al* (1991) *Bio/Technology* 9, 183-187.

Tanguy-Rougeau, C. *et al* (1988) *FEBS Lett.* 234, 464-470

Wang, H.Y. *et al* (1979) *Biotech. & Bioeng.* 21, 975.

10 Winston, F. *et al* (1983) *Methods Enzymol.* 101, 211-228.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Delta Biotechnology Limited
- (B) STREET: Castle Court, Castle Boulevard
- (C) CITY: Nottingham
- (D) STATE: Nottinghamshire
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast strains and modified albumins

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 73..1827

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAGTGGG TAAGCTTTAT TTCCCTTCTT TTTCTCTTTA GCTCGGCTTA TTCCAGGAGC	60
TTGGATAAAA GA GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA	108
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys	
1 5 10	
GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT GCC TTT GCT	156
Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala	
15 20 25	
CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT	204
Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn	
30 35 40	
GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA	252
Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu	
45 50 55 60	

AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr 65 70 75	300
GTT GCA ACT CTT CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA Val Ala Thr Leu Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala 80 85 90	348
AAA CAA GAA CCT GAG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC Lys Gln Glu Pro Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp 95 100 105	396
AAC CCA AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys 110 115 120	444
ACT GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr 125 130 135 140	492
GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe 145 150 155	540
TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA GCT GCT Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala 160 165 170	588
GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA Asp Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu 175 180 185	636
GGG AAG GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT GCC AGT CTC CAA Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln 190 195 200	684
AAA TTT GGA GAA AGA GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser 205 210 215 220	732
CAG AGA TTT CCC AAA GCT GAG TTT GCA GAA GTT TCC AAG TTA GTG ACA Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr 225 230 235	780
GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT GGA GAT CTG CTT GAA Asp Leu Thr Lys Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu 240 245 250	828
TGT GCT GAT GAC AGG GCG GAC CTT GCC AAG TAT ATC TGT GAA AAT CAA Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln 255 260 265	876
GAT TCG ATC TCC AGT AAA CTG AAG GAA TGC TGT GAA AAA CCT CTG TTG Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu 270 275 280	924
GAA AAA TCC CAC TGC ATT GCC GAA GTG GAA AAT GAT GAG ATG CCT GCT Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala 285 290 295 300	972
GAC TTG CCT TCA TTA GCT GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys 305 310 315	1020
AAA AAC TAT GCT GAG GCA AAG GAT GTC TTC CTG GGC ATG TTT TTG TAT Lys Asn Tyr Ala Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr 315	1068

320	325	330	
GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA Glu Tyr Ala Arg Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg 335 340 345			1116
CTT GCC AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA Leu Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala 350 355 360			1164
GAT CCT CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu 365 370 375 380			1212
GTG GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG Val Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu 385 390 395			1260
CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTA TTA GTT CGT TAC ACC Gln Leu Gly Glu Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr 400 405 410			1308
AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC TCA AGA Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg 415 420 425			1356
AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT CCT GAA GCA AAA Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys 430 435 440			1404
AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC CTG AAC CAG TTA Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu 445 450 455 460			1452
TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT GAC AGA GTC ACC AAA TGC Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys 465 470 475			1500
TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA GCT CTG GAA Cys Thr Glu Ser Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu 480 485 490			1548
GTC GAT GAA ACA TAC GTT CCC AAA GAG TTT AAT GCT GAA ACA TTC ACC Val Asp Glu Thr Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr 495 500 505			1596
TTC CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys 510 515 520			1644
AAA CAA ACT GCA CTT GTT GAG CTC GTG AAA CAC AAG CCC AAG GCA ACA Lys Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr 525 530 535 540			1692
AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT TTC GCA GCT TTT GTA GAG Lys Glu Gln Leu Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu 545 550 555			1740
AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT Lys Cys Cys Lys Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly 560 565 570			1788
AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA Lys Lys Leu Val Ala Ala Ser Gln Ala Ala Leu Gly Leu 575 580 585			1830

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 585 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
 1           5           10           15
Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
          20           25           30
Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
          35           40           45
Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
          50           55           60
Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
          65           70           75           80
Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
          85           90           95
Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
          100          105          110
Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
          115          120          125
Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
          130          135          140
Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
          145          150          155          160
Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
          165          170          175
Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
          180          185          190
Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
          195          200          205
Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
          210          215          220
Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
          225          230          235          240
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
          245          250          255
Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
          260          265          270
Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
          275          280          285
Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser

```


290	295	300
Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala		
305	310	315 320
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg		
	325	330 335
Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr		
	340	345 350
Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu		
	355	360 365
Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro		
	370	375 380
Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu		
	385	390 395 400
Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro		
	405	410 415
Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys		
	420	425 430
Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys		
	435	440 445
Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His		
	450	455 460
Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser		
	465	470 475 480
Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr		
	485	490 495
Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp		
	500	505 510
Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala		
	515	520 525
Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu		
	530	535 540
Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys		
	545	550 555 560
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val		
	565	570 575
Ala Ala Ser Gln Ala Ala Leu Gly Leu		
	580	585

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Tyr Thr Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Tyr Thr Gln Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCAGACCT TGCATGCAGC CAAGACACCC TCACATAGC

39

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTTACGTT CTGTGGTGGC ATGCCCACCTT CCAAGTCCAC CG

42

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTCTCATA GTGGAAAAGC TTCTAAATAC GACAACTTCC CC

42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCAAAATGG TACCTGTGTC ATCACTCGTT GGGATAATAC C

41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTGGAT CCAATGGTGC TTTGGCCAAA TAAATAGTTT CAGC

44

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTTCTTTTA CCGGTAACAA GCTTGAGTCC ATTGG

35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTAAGGTTT AGTCGACCTA TTTTGTGTTT TGTCTGC

37

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGAAACGTAT GAATTCGATA TCATTGATAC AGACTCTGAG TACG

44

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys
 1 5 10 15

Ile Ser Ala Asp Ala His Lys Ser
 20

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4106 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAATTCTCTG TTGACTACTA AACTGAGAGA ATTTGCCGAG ACTCTAAGAA CAGCTTTGAA	60
AGAGCGTTCT GCCGATGATT CCATAATTGT CACTCTGAGA GAGCAAATGC AAAGAGAAAT	120
CTTCAGGTTG ATGTCGTTGT TCATGGACAT ACCTCCAGTG CAACCAAACG AGCAATTCAC	180
TTGGGAATAC GTTGACAAAG ACAAGAAAAT CCACACTATC AAATCGACTC CGTTAGAATT	240
TGCCTCCAAA TACGCAAAT TGGACCCTTC CACGCCAGTC TCATTGATCA ATGATCCAAG	300
ACACCATATG GTAAATTAAT TAAGATCGAT CGTTTAGGAA ACGTCCTTGG CGGAGATGCC	360
GTGATTTACT TAAATGTTGA CAATGAAACA CTATCTAAAT TGGTTGTTAA GAGATTACAA	420
AATAACAAAG CTGTCTTTTT TGGATCTCAC ACTCCAAAGT TCATGGACAA GAAAACTGGT	480
GTCATGGATA TTGAATTGTG GAACTATCCT GCCATGGCTA TAATTTACCT CAGCAAAAGG	540
CATCCGGTAT TAGATACCAT GAAAGTTTGA TGAATCATGC TATGTTGGAT CACTGGCTGC	600
CACGTCGATG AAACGTCTAA ATTACCACTT CGCTACCGTC TGAAAATTCC TGGGGTAAAG	660

ACTCCGGTAA	AGACGGATTA	TACGTGATGA	CTCAAAAGTA	CTTCGAGGAG	TACTGCTTTC	720
AAATTGTGGT	CGATATCAAT	GAATTGCCAA	AAGAGCTGGC	TTCAAAATTC	ACCTCAGGTA	780
AGGAAGAGCC	GATTGTCTTG	CCCATCTGGA	CCCAATGGTG	CTTTGGCCAA	ATAAATAGTT	840
TCAGCAGCTC	TGATGTAGAT	ACACGTATCT	CGACATGTTT	TATTTTACT	ATACATACAT	900
AAAAGAAATA	AAAAATGATA	ACGTGTATAT	TATTATTCAT	ATAATCAATG	AGGGTCATTT	960
TCTGAAACGC	AAAAAACGGT	AAATGGAAAA	AAAATAAAGA	TAGAAAAAGA	AAACAAACAA	1020
AGGAAAGGTT	AGCATATTAA	ATAACTGAGC	TGATACTTCA	ACAGCATCGC	TGAAGAGAAC	1080
AGTATTGAAA	CCGAAACATT	TTCTAAAGGC	AAACAAGGTA	CTCCATATTT	GCTGGACGTG	1140
TTCTTTCTCT	CGTTTCATAT	GCATAATTCT	GTCATAAGCC	TGTTCTTTTT	CCTGGCTTAA	1200
ACATCCCGTT	TTGTAAAAGA	GAAATCTATT	CCACATATTT	CATTCATTCTG	GCTACCATAC	1260
TAAGGATAAA	CTAATCCCGT	TGTTTTTTGG	CCTCGTCACA	TAATTATAAA	CTACTAACCC	1320
ATTATCAGAT	GAAAGTGAGG	AAATATATTA	CTTTATGCTT	TTGGTGGGCC	TTTTCAACAT	1380
CCGCTCTTGT	ATCATCACAA	CAAATTCAT	TGAAGGACCA	TACGTCACGA	CAGTATTTTG	1440
CTGTAGAAAG	CAATGAAACA	TTATCCCGCT	TGGAGGAAAT	GCATCCAAAT	TGGAAATATG	1500
AACATGATGT	TCGAGGGCTA	CCAAACCATT	ATGTTTTTTC	AAAAGAGTTG	CTAAAATTGG	1560
GCAAAGATC	ATCATTAGAA	GAGTTACAGG	GGGATAACAA	CGACCACATA	TTATCTGTCC	1620
ATGATTTATT	CCCGCGTAAC	GACCTATTTA	AGAGACTACC	GGTGCCTGCT	CCACCAATGG	1680
ACTCAAGCTT	GTTACCGGTA	AAAGAAGCTG	AGGATAAACT	CAGCATAAAT	GATCCGCTTT	1740
TTGAGAGGCA	GTGGCACTTG	GTCAATCCAA	GTTTTCTGG	CAGTGATATA	AATGTTCTTG	1800
ATCTGTGGTA	CAATAATATT	ACAGGCGCAG	GGGTCGTGGC	TGCCATTGTT	GATGATGGCC	1860
TTGACTACGA	AAATGAAGAC	TTGAAGGATA	ATTTTTGCGC	TGAAGGTTCT	TGGGATTTCA	1920
ACGACAATAC	CAATTTACCT	AAACCAAGAT	TATCTGATGA	CTACCATGGT	ACGAGATGTG	1980
CAGGTGAAAT	AGCTGCCAAA	AAAGGTAACA	ATTTTTGCGG	TGTCGGGGTA	GGTTACAACG	2040
CTAAAATCTC	AGGCATAAGA	ATCTTATCCG	GTGATATCAC	TACGGAAGAT	GAAGCTGCGT	2100
CCTTGATTTA	TGGTCTAGAC	GTAAACGATA	TATATTCATG	CTCATGGGGT	CCCGCTGATG	2160
ACGGAAGACA	TTTACAAGGC	CCTAGTGACC	TGGTGAAAAA	GGCTTTAGTA	AAAGGTGTTA	2220
CTGAGGGAAG	AGATTCCAAA	GGAGCGATTT	ACGTTTTTGC	CAGTGGAAT	GGTGGAAGTC	2280
GTGGTGATAA	TTGCAATTAC	GACGGCTATA	CTAATTCCAT	ATATTCTATT	ACTATTGGGG	2340
CTATTGATCA	CAAAGATCTA	CATCCTCCTT	ATTCCGAAGG	TTGTTCCGCC	GTCATGGCAG	2400
TCACGTATTC	TTCAGGTTCA	GGCGAATATA	TTCATTCGAG	TGATATCAAC	GGCAGATGCA	2460
GTAATAGCCA	CGGTGGAACG	TCTGCGGCTG	CTCCATTAGC	TGCCGGTGTT	TACACTTTGT	2520
TACTAGAAGC	CAACCCAAAC	CTAACTTGGA	GAGACGTACA	GTATTTATCA	ATCTTGTCTG	2580
CGGTAGGGTT	AGAAAAGAAC	GCTGACGGAG	ATTGGAGAGA	TAGCGCCATG	GGGAAGAAAT	2640

ACTCTCATCG CTATGGCTTT GGTAAAATCG ATGCCCATAA GTTAATTGAA ATGTCCAAGA	2700
CCTGGGAGAA TGTTAACGCA CAAACCTGGT TTTACCTGCC AACATTGTAT GTTTCCCAGT	2760
CCACAAACTC CACGGAAGAG ACATTAGAAT CCGTCATAAC CATATCAGAA AAAAGTCTTC	2820
AAGATGCTAA CTTCAAGAGA ATTGAGCACG TCACGGTAAC TGTAGATATT GATACAGAAA	2880
TTAGGGGAAC TACGACTGTC GATTTAATAT CACCAGCGGG GATAATTTCA AACCTTGGCG	2940
TTGTAAGACC AAGAGATGTT TCATCAGAGG GATTCAAAGA CTGGACATTC ATGTCTGTAG	3000
CACATTGGGG TGAGAACGGC GTAGGTGATT GGAAAATCAA GGTTAAGACA ACAGAAAATG	3060
GACACAGGAT TGACTTCCAC AGTTGGAGGC TGAAGCTCTT TGGGGAATCC ATTGATTCAT	3120
CTAAAACAGA AACTTTCGTC TTTGGAAACG ATAAAGAGGA GGTGTAACCA GCTGCTACAG	3180
AAAGTACCGT ATCACAATAT TCTGCCAGTT CAACTTCTAT TTCCATCAGC GCTACTTCTA	3240
CATCTTCTAT CTCAATTGGT GTGGAAACGT CGGCCATTCC CCAAACGACT ACTGCGAGTA	3300
CCGATCCTGA TTCTGATCCA AACACTCCTA AAAAATTTTC CTCTCCTAGG CAAGCCATGC	3360
ATTATTTTTT AACAAATATT TTGATTGGCG CCACATTTTT GGTGTTATAC TTCATGTTTT	3420
TTATGAAATC AAGGAGAAGG ATCAGAAGGT CAAGAGCGGA AACGTATGAA TTCGATATCA	3480
TTGATACAGA CTCTGAGTAC GATTCTACTT TGGACAATGG AACTTCCGGA ATTACTGAGC	3540
CCGAAGAGGT TGAGGACTTC GATTTTGATT TGTCCGATGA AGACCATCTT GCAAGTTTGT	3600
CTTCATCAGA AAACGGTGAT GCTGAACATA CAATTGATAG TGTACTAACA AACGAAAATC	3660
CATTTAGTGA CCCTATAAAG CAAAAGTTCC CAAATGACGC CAACGCAGAA TCTGCTTCCA	3720
ATAAATTACA AGAATTACAG CCTGATGTTT CTCCATCTTC CGGACGATCG TGATTCGATA	3780
TGTACAGAAA GCTTCAAATT ACAAATAGC ATTTTTTCT TATAGATTAT AATACTCTCT	3840
CATACGTATA CGTATATGTG TATATGATAT ATAAACAAAC ATTAATATCC TATTCCTTCC	3900
GTTTGAAATC CCTATGATGT ACTTTGCATT GTTTGCACCC GCGAATAAAA TGAAAATCC	3960
GAACCGATAT ATCAAGCACA TAAAGGGGA GGGTCCAATT AATGCATATT TAAGACCACA	4020
GCTGAATAAC TTTAAAACGG CAGACAAAAC AAAAAATAGG TCGAATAAAC CTTACCTGCC	4080
TAGAAGGAAT GACAGCAGCT AATAAG	4106

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2526 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCGTTTTCTT TTCGTAAAAA AAAACAATAG ACACTATATA TAGACACTTT TTCCTTTCCT	60
TCTTTGCGCG ATTTCAAGAG GAAAAGCATA CTAAATAAG AATATTCCTA AAACACACGT	120
TCTGACGCGT CAATTAGATC GTCAGACCTT GCATGCAGCC AAGACACCCT CACATAGCAC	180
TGCCTCCTTC CTCCTCTTTT CTGTCACCAC CTCACCTCCC TCGTCCACTC AACTGAGTGG	240
CTTTTCGCTC CTTTATACT GCGCCATGAG TAGTTTTCGT TTCACTGATG TGTCCGAAAA	300
AATTGAGGTT TCATAAAAAA ATTCGTGGAC TTATTTATGG AGAAACAGGG AAATCCGACT	360
ACTTAAGAAA AGGGTGTCAA AGAGGATTTA CTTTTTTCCT TCTTTTGTGA TTTGTTCCCTA	420
TTTCCGCAAT TGGACGGTTA TTAAGAAGAA CGCAATTGGC TTTTCTGTAT ATTAAAATAC	480
ATAGCGTAAT AAAAAGATAA GGTGAACACC AAGCATATAG TATAATATTA CCTACCACAT	540
ATGAAACTGA AAAGTGTAA ATCTGCGGTC CTTTCGTCAC TCTTTGCATC GCAGGTTCTC	600
GGTAAGATAA TACCAGCAGC AAACAAGCGC GACGACGACT CGAATTCCAA GTTCGTCAAG	660
TTGCCCTTTC ATAAGCTTTA CGGGGACTCG CTAGAAAATG TGGGAAGCGA CAAAAAACCG	720
GAAGTACGCC TATTGAAGAG GGCTGACGGT TATGAAGAAA TTATAATTAC CAACCAGCAA	780
AGTTTCTATT CGGTGGACTT GGAAGTGGGC ACGCCACCAC AGAACGTAAC GGTCTGGTG	840
GACACAGGCT CCTCTGATCT ATGGATTATG GGCTCGGATA ATCCATACTG TTCTTCGAAC	900
AGTATGGGTA GTAGCCGGAG ACGTGTTATT GACAAACGTG ATGATTCGTC AAGCGGCGGA	960
TCTTTGATTA ATGATATAAA CCCATTTGGC TGGTTGACGG GAACGGGCAG TGCCATTGGC	1020
CCCACTGCTA CGGGCTTAGG AGGCGGTTCA GGTACGGCAA CTCAATCCGT GCCTGCTTCG	1080
GAAGCCACCA TGGACTGTCA ACAATACGGG ACATTTTCCA CTTCGGGCTC TTCTACATTT	1140
AGATCAAACA ACACCTATTT CAGTATTAGC TACGGTGATG GGACTTTTGC CTCCGGTACT	1200
TTTGGTACGG ATGTTTTGGA TTTAAGCGAC TTGAACGTTA CCGGGTTGTC TTTTGCCGTT	1260
GCCAATGAAA CGAATTCTAC TATGGGTGTG TTAGGTATTG GTTTGCCCCG ATTAGAAGTC	1320
ACTTATTCTG GCTCTACTGC GTCTCATAGT GGAAAAGCTT ATAAATACGA CAACTTCCCC	1380
ATTGTATTGA AAAATTCTGG TGCTATCAAA AGCAACACAT ATTCTTTGTA TTTGAACGAC	1440
TCGGACGCTA TGCATGGCAC CATTTTGTTT GGAGCCGTGG ACCACAGTAA ATATACCGGC	1500
ACCTTATACA CAATCCCCAT CGTAAACACT CTGAGTGCTA GTGGATTTAG CTCTCCCATT	1560
CAATTTGATG TCACTATTAA TGGTATCGGT ATTAGTGATT CTGGGAGTAG TAACAAGACC	1620
TTGACTACCA CTAAAATACC TGCTTTGTCT GATTCCGGTA CTACTTTGAC TTATTTACCT	1680
CAAACAGTGG TAAGTATGAT CGCTACTGAA CTAGGTGCGC AATACTCTTC CAGGATAGGG	1740
TATTACGTAT TGGACTGTCC ATCTGATGAT AGTATGGAAA TAGTGTTCTG TTTTGGTGGT	1800
TTTCACATCA ATGCACCACT TTCGAGTTTT ATCTTGAGTA CTGGCACTAC ATGTCTTTTA	1860
GGTATTATCC CAACGAGTGA TGACACAGGT ACCATTTTGG GTGATTCATT TTTGACTAAC	1920

GCGTACGTGG	TTTATGATTT	GGAGAATCTT	GAAATATCCA	TGGCACAAGC	TCGCTATAAT	1980
ACCACAAGCG	AAAATATCGA	AATTATCACA	TCCTCTGTTC	CAAGCGCCGT	AAAGGCACCA	2040
GGCTATACAA	ACACTTGGTC	CACAAGTGCA	TCTATTGTTA	CCGGTGGTAA	CATATTTACT	2100
GTAAATTCCT	CACAAACTGC	TTCTTTTAGC	GGTAACCTGA	CGACCAGTAC	TGCATCCGCC	2160
ACTTCTACAT	CAAGTAAAAG	AAATGTTGGT	GATCATATAG	TTCCATCTTT	ACCCCTCACA	2220
TTAATTTCTC	TTCTTTTTGC	ATTCATCTGA	AAACCGTTGC	ACAAAGTTTA	GACATTCACA	2280
TCTCCAAGCC	AGTTGGAGTT	TCTGGCGGAA	ATCGTTGCTC	TCGCTTGGGC	AAAGTTTTTT	2340
TTTATTATTA	ATTTTTTATT	GTTACGTTGG	CGGTCTTTAT	TTTTACTTCA	CAATAGTTTA	2400
TCTTACCCAC	TAAGAATAGG	TTACCATTTA	TTACATTTT	TTTTTCTCAT	TCCTAGTATA	2460
CTATTTACCT	GGGATATGGC	CTATAATCAA	AGGCTTTAAT	ATTCTAATAA	TTCGTTTGGC	2520
ATCTAG						2526

CLAIMS

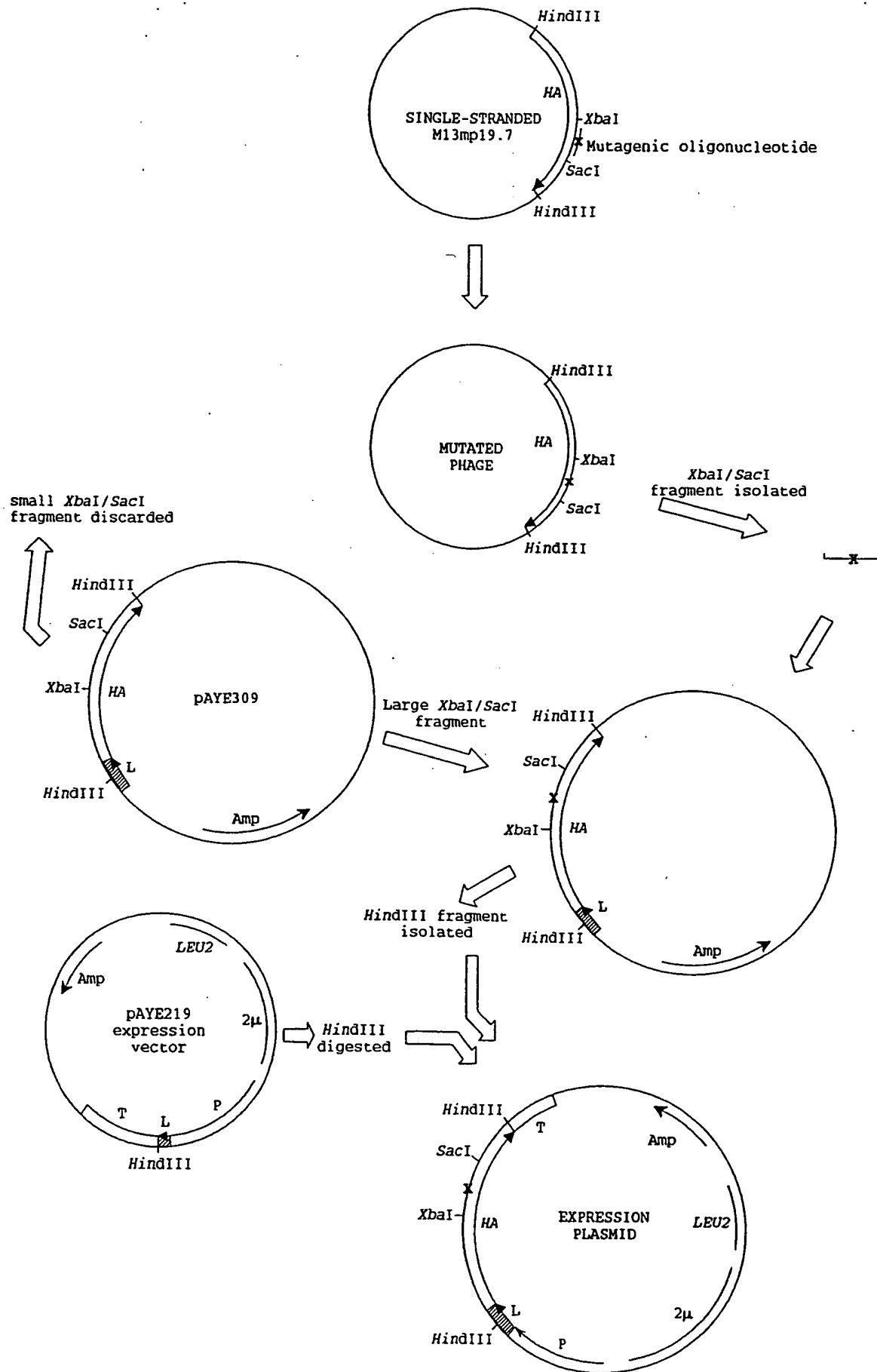
1. A process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.
2. A process according to Claim 1 wherein the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.
3. A process according to Claim 1 or 2 wherein the yeast is *S. cerevisiae*.
4. A process according to Claim 1, 2 or 3 wherein the yeast lacks a functional *YAP3* gene or homologue thereof.
5. A process according to any one of Claims 1 to 4 wherein the yeast cells additionally have a reduced level of *S. cerevisiae* Kex2p proteolytic activity.
6. A process according to any one of the preceding claims wherein the albumin is a human albumin.
7. A culture of yeast cells containing a polynucleotide sequence encoding an albumin and a second polynucleotide sequence encoding a secretion signal causing albumin expressed from the first polynucleotide sequence to be secreted from the yeast, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic

activity.

8. A culture according to Claim 7 wherein the albumin is a human albumin.
- 5 9. A culture according to Claim 7 or 8 wherein the yeast is *S. cerevisiae*.
- 10 10. A culture according to any one of Claims 7 to 9 wherein the said signal is cleaved by the yeast prior to release of the albumin from the yeast.
11. A culture according to any one of Claims 7 to 10 wherein the yeast cells additionally have a reduced level of Kex2p proteolytic activity.
- 15 12. A culture according to Claim 11 wherein the said secretion signal is cleaved from the albumin by a protease other than Kex2p.
- 20 13. A modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with yeast aspartyl protease 3 (Yap3p) when expressed and secreted in yeast, characterised in that the modified albumin is not susceptible to such cleavage.
- 25 14. A modified albumin according to Claim 13 wherein the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein.
- 30 15. A modified albumin according to Claim 13 or 14 wherein the said monobasic amino acid is arginine.

16. A modified albumin according to Claim 14 or 15 wherein the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin.
- 5 17. A modified albumin according to Claim 16 wherein the said pair of amino acids is Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg.
- 10 18. A modified albumin according to Claim 13 wherein the naturally-occurring albumin is a human albumin and the modified protein lacks Arg⁴¹⁰; and, optionally, residues 413 and 414 do not each consist of lysine or arginine.
- 15 19. A modified albumin according to Claim 18 which is a human albumin having the amino acid changes R410A, K413Q, K414Q.
20. A polynucleotide encoding a modified albumin according to any one of Claims 13 to 19.
- 20 21. A yeast containing a polynucleotide according to Claim 20, transcription signals such that the modified albumin is expressed in the yeast, and a further polynucleotide adjacent the said polynucleotide such that the modified albumin is secreted from the yeast.

1/5
Figure 1.



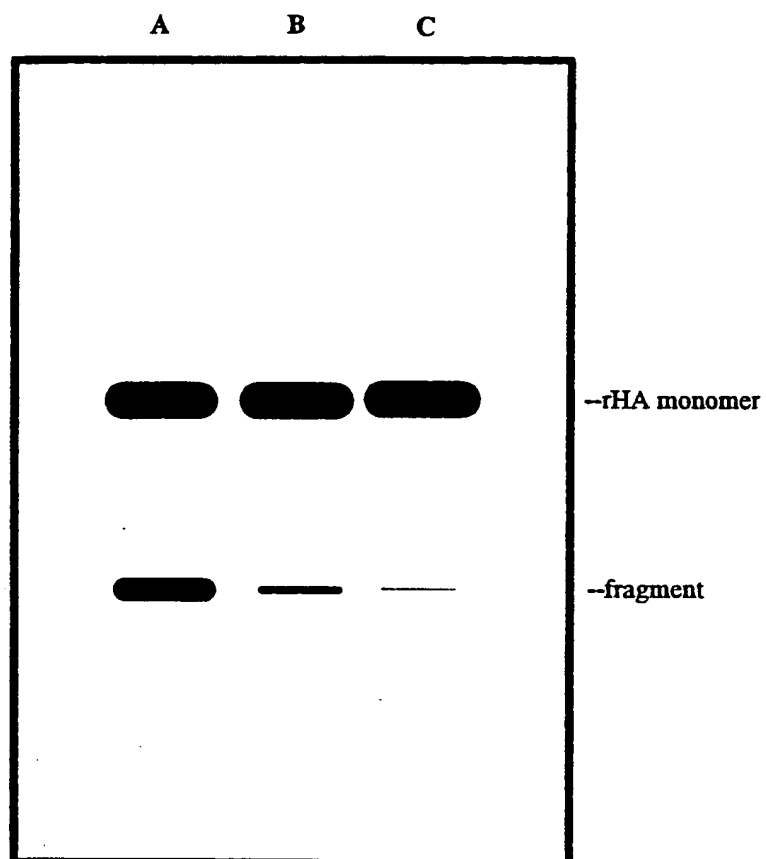


Figure 3

5' and 3' regions of YAP3 obtained by PCR:

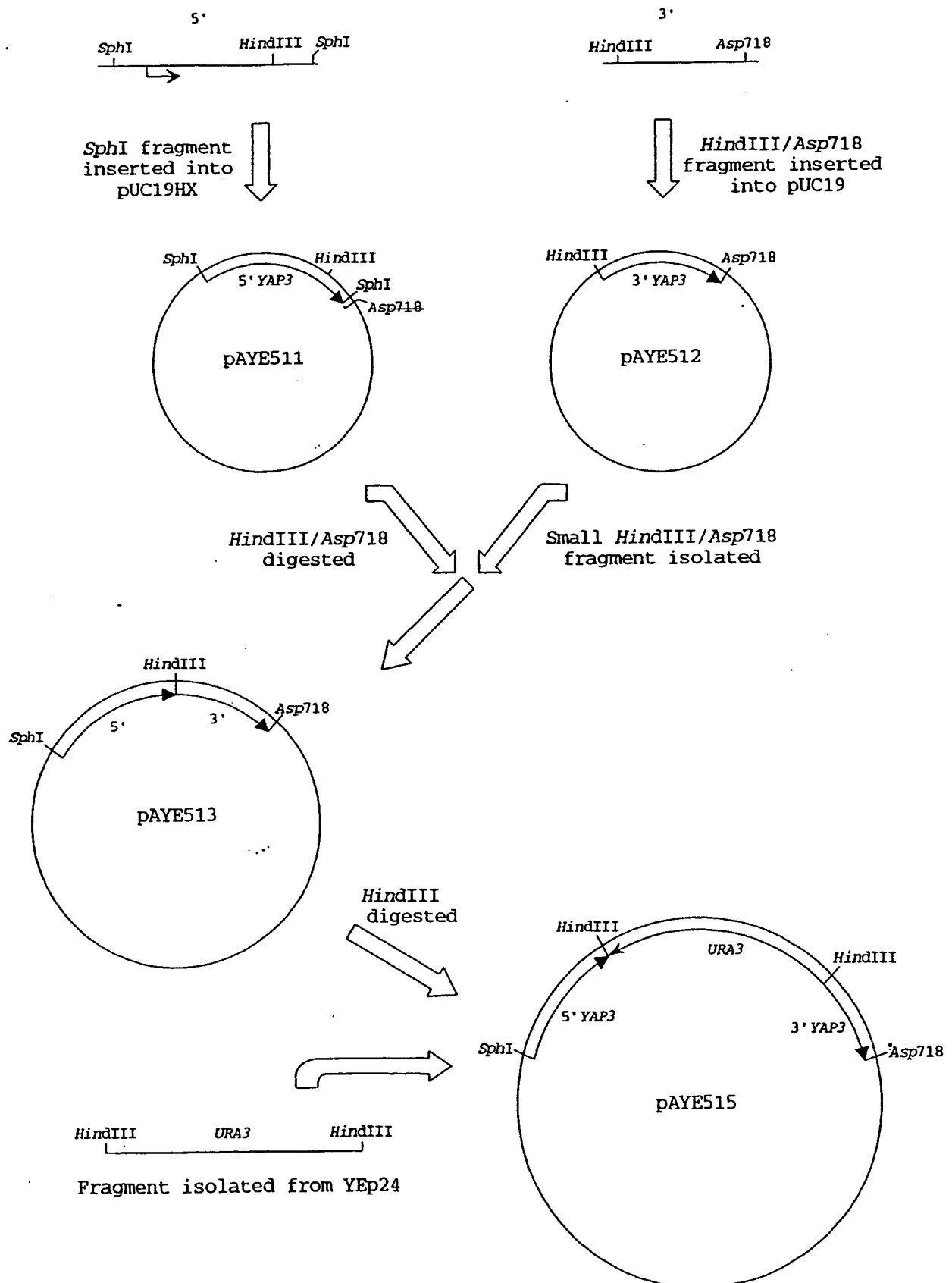
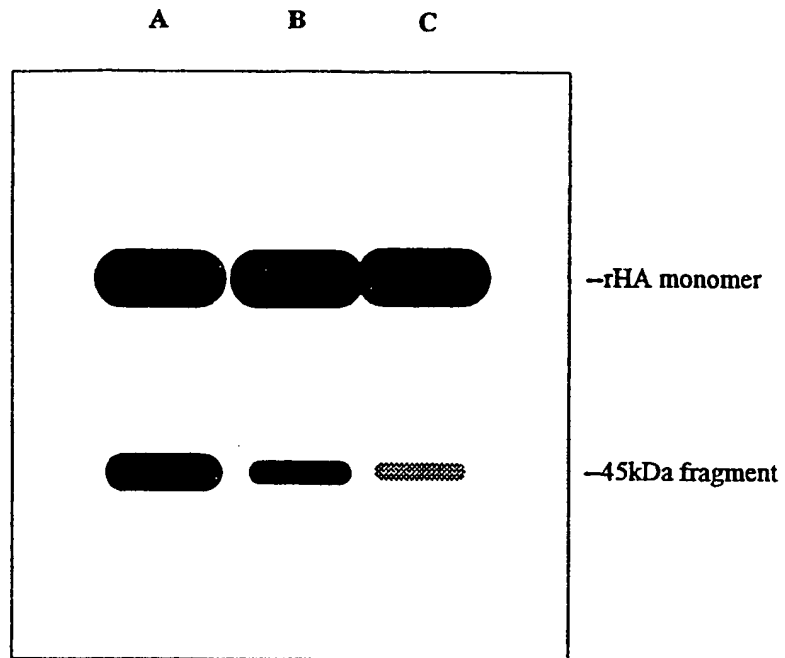
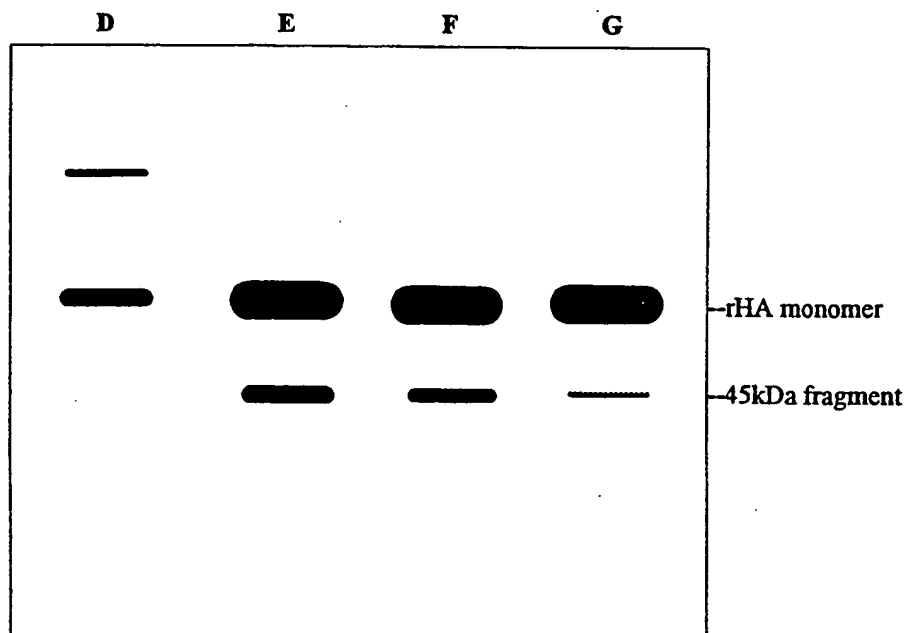
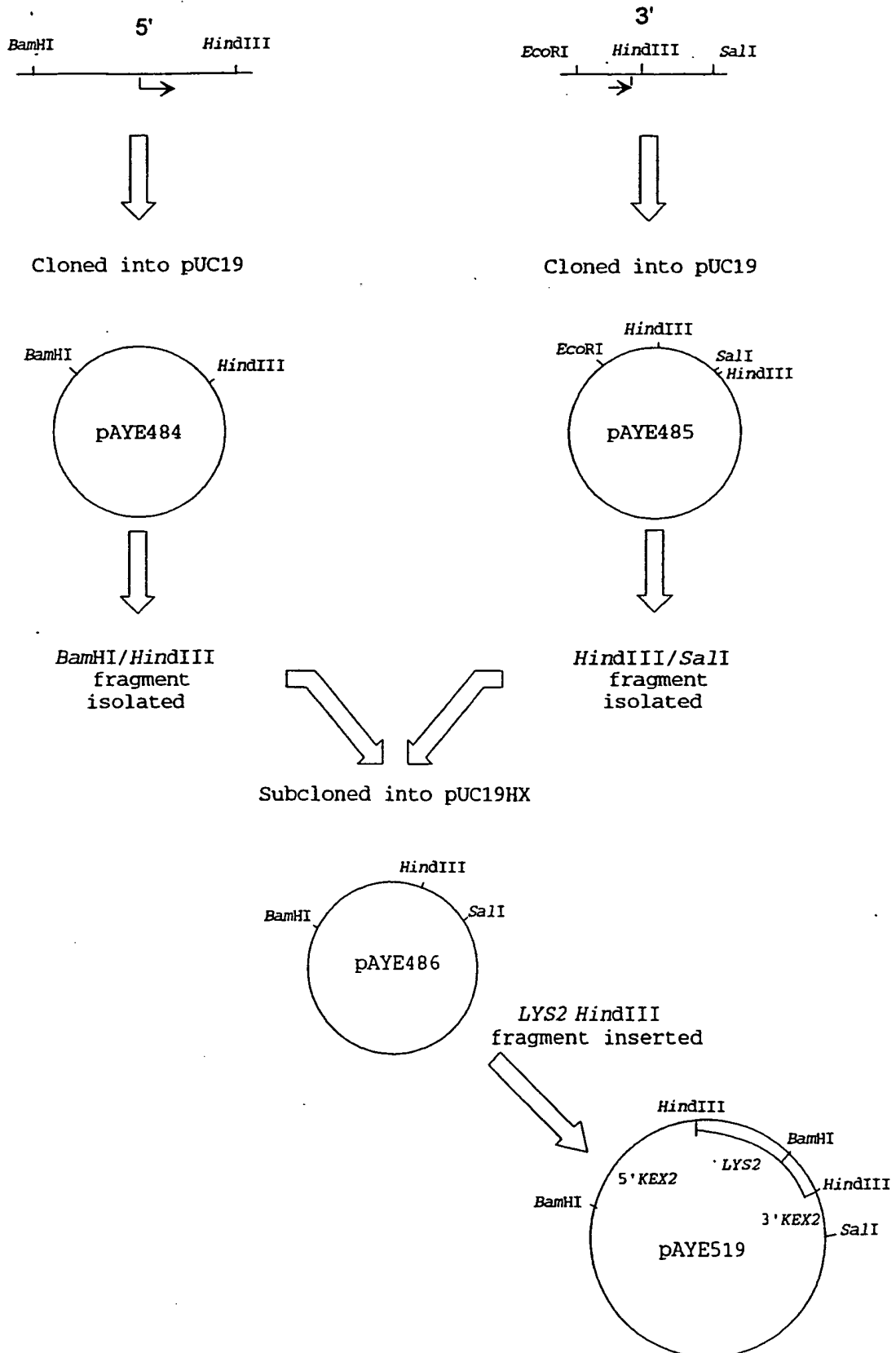


Figure 4Figure 5

5/5
Figure 6

5' and 3' regions of KEX2 obtained by PCR:



A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N1/21 A61K38/38 C12P21/02 C12N15/11
C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	BIOCHIMIE, vol. 76, 1994 pages 226-233, Y. BOURBONNAIS ET AL. 'Cleavage of prosomatostatins by the yeast Yap3 and Kex2 endoprotease' *see the whole article* ---	1-21
Y	BIOTECHNOLOGY, vol. 8, no. 1, 1990 pages 42-46, D. SLEEP ET AL. 'The secretion of human serum albumin from the yeast S. cerevisiae using five different leader sequences' *see the whole article* --- -/--	1-21

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

23 June 1995

Date of mailing of the international search report

- 5. 07. 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Marie, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL, vol. 12, no. 1, 1993 pages 285-294, Y. BOURBONNAIS ET AL. 'Isolation and characterisation of S. cerevisiae mutants defective in somatostatin expression...' *see the whole article* ---	1-21
Y	YEAST, vol. 6, 1990 pages 127-137, M. EGEL-MITANI ET AL. 'A novel aspartyl protease allowing KEX2-independent MF alfa propheromone processing in yeast' *see the whole article* -----	1-21

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.